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Advanced detection methods for control of plant bacterial pathogens

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Executive Summary

The aim of this project was to develop specific and sensitive PCR-based procedures for routine detection of two economically important plant pathogenic bacteria in cabbage seeds, *Xanthomonas campestris* pv. *campestris* (*Xcc*) and *Erwinia carotovora* subsp. *carotovora* (*Ecc*). Bacterial diseases of plants are responsible for heavy losses to many crops in Kazakhstan and other Central Asian Republics. They attack vegetables, ornamental or fruits in the field and during shipment and storage. In cabbage, losses due to *Xcc* and *Ecc* can reach 80%. Phytobacteriology in Kazakhstan is not well developed and diagnostic procedures are based primarily on some classical methods such as isolation on simple media and pathogenicity tests. During the project modern diagnostic procedures have been introduced to the Kazakh researchers that were trained at the Volcani Center in Bet Dagan. The latter involved isolation of the pathogens on semi-selective media from different regions in Kazakhstan, isolation of DNA, performance of PCR with arbitrary primers, selection of specific primers, performance of PCR with specific primers and determination of the threshold level for bacterial detection in cabbage seeds. Furthermore, all the necessary equipment and materials required for molecular diagnostic procedures were purchased for the Kazakh laboratories. The development and/or utilization of PCR-based procedures for detection of *Xcc* and *Ecc* in cabbage seeds, in collaboration with the Kazakh researchers, will allow them to implement, independently, similar procedures with other important bacterial pathogens in the future.

Research Objectives

Black rot, caused by *Xanthomonas campestris* pv. *campestris* (*Xcc*) is considered the most important disease of crucifers occurring worldwide on all cultivated brassicas and radishes and no numerous cruciferous weeds (Williams 1980). Cabbage is a major crop grown in all regions of Kazakhstan (Jamurzina 1993). The extend of plant mortality in Kazakhstan due to *Xcc* is about 40% and with the infection by *Erwinia carotovora* subsp. *carotovora* (*Ecc*) that generally invade due to the black rot symptoms, may often reach 80-100% (Jamurzina 1993). In Israel *Xcc* infects radish, cabbage, cauliflower, mustard, horseradish, broccoli and other brassicas particularly in the coast region (Volcani 1985). *Xcc* is seedborne and moves systemically in the plant after germination.

Ecc causes soft rot in many different plant species including cabbage and cauliflower (Volcani 1985). It can attack in the field and during storage or shipping. In Kazakhstan the combination of *Ecc* and *Xcc* in cabbage is devastating and the entire filed can be wiped out.

In the present project we intended to obtain useful tools and acquire significant information for developing effective control measures against these two bacteria. Since the most important source of primary inoculum in case of *Xcc* is infected seeds, our efforts have been aimed at developing tools for obtaining pathogen-free seeds. Although the infection by *Ecc* could be considered secondary to the primary infection by *Xcc*, cabbage seeds infested with *Ecc* were detected in Israel and can severely aggravate the damage caused by *Xcc*. Therefore efforts in this study were aimed at developing advanced procedures for detection of *Xcc* and *Ecc* in crucifer seeds. Although a number of diagnostic tests have been described for *Xcc* and *Ecc* detection (e.g., Alvarez et al. 1994, Schaad 1989, Klopmeyer et al. 1988, Darrasse et al. 1994), a reproducible, sensitive, rapid and specific detection procedure which will also replace pathogenicity test was still needed. Our approach was to establish protocols based on PCR for detection of *Xcc* and *Ecc* in crucifer seeds since PCR has the advantage of specificity, sensitivity and ability to detect the pathogen in a short time.

Before this project started effective procedures for detection of phytobacterial pathogens in Kazakhstan, practically, did not exist. Therefore the main purpose of this project was to strengthen the capability of Kazakh researchers in diagnosis of bacteria utilizing *Xcc* and *Ecc* as important models.

Our specific objectives were:

a) Re-assessment of bacterial infection of cabbage in Kazakhstan
b) Development of a specific and sensitive PCR-based method for routine detection of *Xcc* in seeds.

c) Development of a detection procedure for *Ecc* in cabbage seeds by using primers from a pectate lyase gene.

During the course of this project we have developed specific PCR primers for *Xcc* and adapted specific primers for *Ecc*. A most significant part of our study was the development of working protocols for detecting these pathogens in crucifer seeds and assessment of the black rot and soft rot diseases on these hosts in Kazakhstan.

Methods and Results

A. Assessment of bacterial infection of cabbage in Kazakhstan

The assessment of bacterial infection of cabbage in different regions of Kazakhstan has been a major subject of our research during 1998-2000. The fields of north, west, east and southeast of Kazakhstan were visited by us. *Erwinia carotovora* is capable of inducing disease symptoms at temperature below 23°C, which occurs in cool temperate climate, such as Pavlodar. Aktuibinsk that belongs to west Kazakhstan, was chosen as a large agricultural area with numerous cabbage fields. East and southeast, including Semipalatinsk, Djambul, Taldy-Kurgan and almaty regions are traditional cabbage growing area. They are also known to have vegetables which are particularly sensitive to plant pathogens. Cabbage obtained from all these areas was associated with black and soft rot diseases during vegetation, harvest, transit and storage.

Cabbage is traditionally grown by seedlings in the north and west of Kazakhstan. The seedlings are previously grown from seeds in greenhouse starting in the mid of March and are usually being transferred to the field at the end of May. No disease symptoms were observed during seedlings development in the greenhouse and the first disease symptoms were detected in young plants after two weeks growth in the field. The most destructive effect of *Xcc* was observed during July. Soft rot caused by *Ecc* was observed during August-September in the East and Southeast regions.

In the Almaty area cabbage is developed directly from seeds. Since spring of 1998 was cool and rainy, the cabbage was sown in May 10-15 instead of the middle of April. Three varieties of cabbage common for Almaty region were sown: 1. Early varieties, "Iiunskaya"and "Greebovskaya"; 2. Medium varieties, "Podarok", "Zeemovka"and "Belokachannaya"; 3. Late varieties, "Berechukutskaya" and "Zavadovskaya". Early varieties are considered relatively resistant whereas the most sensitive to both bacterial diseases are the late varieties.

We have started to follow the etiology of the disease since 1998. Black rot was initiated by *Xcc* from the leaf edge as yellow spots spreading to the center where the tissue gradually died off. Sometime cabbage heads became distorted or immature. Dark veins were commonly observed on stem sections and leaves.

Soft rot initiated by *Ecc* appeared as rotting of the leaves' surface and spreading later into inner parts of the head. Sometimes no symptoms could be detected but inside of the head the rotting process has already occurred causing plant death.

Isolation of *Xcc* and *Ecc* were performed as follow: Plant material was brought to the laboratory, washed with tap water and small pieces of infected tissue were grounded with pestle and mortar. One drop of the suspension (dilutions 10^2, 10^4, 10^6, 10^8, 10^{10}) was spread on the PDA medium and incubated 2, 3, 4 days at room temperature during summer time and at 26°C during the autumn season. *Xanthomonas* and *Erwinia* colonies with characteristic growth pattern were subsequently transferred to tween or starch containing media, respectively. *Xanthomonas* appeared as circular, raised, yellow colonies after 4 days on tween medium developing white zones around yellow colonies. *Erwinia* appeared as white colonies with dense center and wavy edges. For pathogenicity tests cabbage seedlings with 2-3 true leaves were inoculated with 2-day-old culture by injection. Infection was confirmed by production of necrotic spots. Control plants injected with sterile tap water instead of bacterial suspension were used as control. To confirm the identity of *Ecc* isolates, small surface sterilized potato slices were inoculated with bacterial diluted suspension. Pathogenicity was determined by tissue maceration. Pure cultures were stored with glycerol in small tubes at -21°C. At least 300-400 isolates were collected from each region and about 500 single colonies were identified as *Xcc* or *Ecc*.

The most destructive pathogens were observed in east and southeast of Kazakhstan. Nevertheless, in some farms we were unable to isolate bacteria, which could result from pathogen-free seeds used in this area by professional agronomists. The latter situation usually occurred only in small farms. In large fields disease symptoms were always obvious. The distribution of the pathogens was sporadic. For example, during the expedition to Pavlodar, one person succeeded in obtaining pathological material, whereas the second person that was sent to a different field in the same area, was unable to find infected plants. As expected, July was the best month to collect plants for isolation of *Xanthomonas* in the north and west regions, whereas August-September was

favorable for *Erwinia* development in east and southeast. Nevertheless, in one case we could not isolate bacteria from cabbage with obvious black rot symptoms. One major problem that was often encountered with plant material brought from remote regions, was the fast progress of the infection process. On arrival to Almaty such cabbages were totally rotted. It is therefore possible that the best sources for further collection might be seeds and cabbage from vegetables stores. Isolates exhibited adequate morphological and pathogenic characters were transferred to Israel for further characterization.

During 1999 *Ecc* was isolated from Almaty and Karaganda regions. Isolations were carried out in late June-early July from cabbage plants (during initial formation of the cabbage head). All isolations were made from early varieties of cabbage ("Zimovka" and "Vesnianka"). In the last season low disease severity was observed due to the cold spring temperature that lasted even in July. In August and September, on late varieties of cabbage, we could not find plants with obvious symptoms of soft rot. Instead, many plants were infected with pathogenic fungi. The possibility that clean seeds were used can not be ruled out. Isolations of bacteria were made on PDA plates and strains with characteristic shape and color of *Ecc* were taken for pathogenicity test on potato slices. The strains were transferred to Israel for additional testing namely, growth on CVP and characterization by PCR. None of the strains were positive in both tests.

During the year 2000 only a few cabbage fields were found infected with bacterial diseases. Most of the cabbage growing areas in the Almaty, Djambul and Taldy-Kurgan regions were sown with seeds originated in Holland with tolerance to bacterial diseases. Supposedly for this reason most of cabbage plants looked healthy during the summer and storage. However, cabbage heads from several small farms and private persons showed symptoms of soft rot caused by *Ecc* and black rot caused by *Xcc*. Therefore only limited number of isolates could be obtained from these sources.

Soft rot infected plants exhibited unusual traits; only restricted part of the cabbage head which touched the ground exhibited rotting symptoms that didn't invade into the central part of the head. Bacteria were isolated on PDA plates using dilution method showed heterogeneous phenotype. The obtained isolates exhibited low virulence. Efforts to isolate highly pathogenic *Xcc* were also not successful. Only a few isolates exhibited characteristic growth pattern of *Xcc* on TMB (Schaad, et al. 2001) plates. Generally, *Xcc* isolates had a moderate or low pathogenicity. Central and north parts of Kazakhstan were also visited to find bacterial diseases during harvest time. However, we couldn't recover *Ecc* or *Xcc*.

It might be concluded that soft rot and black rot of cabbage during the last two years wasn't as severe as during 1990-1998.

B. Development of a PCR-detection method for *Xanthomonas campestris* pv. *campestris* in crucifer seeds

During 1998-2000 we have established a collection of *Xcc* strains necessary for developing PCR-based detection procedure. Isolation of *Xcc* was made from infected crucifers plants (e.g. cabbage, broccoli and cauliflower) on 4 semi-selective media: NSCAA, FS, CS20, TMB (Schaad et al. 2001). Isolates of *Xcc* obtained from Israel, Kazakhstan and USA were first subjected to biochemical tests of nitrate reduction, gelatin liquification, starch hydrolysis and lipase activity. Positive reacting strains were subjected to pathogenicity tests. Colonies were grown overnight on YDC agar medium. Pathogenicity was performed on cabbage seedlings by stabbing the petiols of the second and third youngest leaves with needle covered with cells from suspected isolate of *X. campestris*. Plants were incubated at 28°C and typical symptoms (e.g. chlorosis, followed by black veins) were observed after 7-10 days. Table 1 summarized the strains isolated from Kazakhstan and Israel and obtained from other places. As can be observed from this table about 50% of the 57 Israeli isolates tested were non-pathogenic in spite of similar biochemical behavior. All these strains were subjected to RAPD analysis using the non-pathogenic isolates as controls.

Table 1: Strains of *Xanthomonas* used in this study

***Xanthomonas* strains and pathovars**	**Strains**	**Origin**	**Pathogenicity on cabbage**
X. campestris pv. *campestris*	602, 528/2, BR1, BR2, CRI-1, CRI-2, CRII-1, CRII-2, 803/1, 803/2, 1338, 1467, CR-5, CR-6, CR-7, CR-8, CR-9, CR-10, 2559/C, 3896/1, 3896/3, XCC422, CB-1, CB-2, CL-1, R-90, R-92	Israel	+
	K4, K8, K151, K101, K124, K117, K112	Kazakhstan (Semipalatinsk)	+
	K1, K2, K7, K21, K22a, K5, K12, K17-1, K11-2, K9a	Kazakhstan (Almaty)	+
	K93/1, K81, K166, K139, K181, K199,	Kazakhstan (Aktiubinsk)	+
	K67, K64, K78, K69-3, K61-11, K69, K72	Kazakhstan (Djambul)	+
	K251, K413, K207, K213, K232	Kazakhstan (Pavlodar)	+
	K41a, K55a, K46, K36, K48-2,	Kazakhstan (Taldy-Kurgan)	+
	Hasib, GAC-20, Cabaret X3, GAC-17, PHW-117, G3-38A, GAC-137, G2-12, G2-17, EEXC-114, A-249, 528/1	USA	+
alfalfae	KX-1	USA	NP
begoniae	077-3382	"	"
cyamopsidis	13D5	"	"
esculenti	084-1093	"	"
glycines	ATCC 17915	"	"
glycines	YR15	Indonesia	"
malvacearum	TX84	USA	"
malvacearum-hiviscus	X108, M84-11	"	"
vesicatoria	592	Israel	"
vignicola	A81-331	USA	"
X. albilineans	635, Xalb	"	"
X. phaseoli	LB-2, XP-JL	"	"
Xanthomonas spp.			"
	K19	Kazakhstan	"
	385, 418, 2855/4, 1411, 2013/1, 2853/4, 2855/2, 3056/a, 3056/g, 3057/c, 3057/g, 3058/g, 3058/a, 3059/7, 3059/8, 3060/g, 3060/h, 3060/c, 3356/2, 2013, R-49, R-71, R-103, R-105, R-110, R-41, R-102, R-51, 3994, 3458	Israel	"

Total DNA was isolated from all the strains listed in Table 1 using kit of Boehringer Mannheim (Cat. No. 1796828). For RAPD analysis, PCR amplifications were carried out in 25 µl volumes and contained 50 ng of genomic DNA, 2 mM $MgCl_2$, 0.4 µM primer, 0.5 U of *Taq* DNA polymerase and 100 µM of each dNTP.

Seventy-seven different arbitrary primers (Operon Technologies) were tested for PCR amplification with pathogenic and non-pathogenic strains. Results in Fig 1 show amplification pattern obtained with primer B13. It can be observed that pathogenic strains produced a specific band of 0.46 kb which was absent in non-pathogenic strains. This fragment was cloned and sequenced. No homology to known genes could be found in the GeneBank. Recently, the whole genome of *Xcc* was sequenced (Da Silva et al. 2002) and the cloned sequence was found to be homologous to an hypothetical protein form *Xcc*. Based on this sequence two specific primers were generated in order to perform a specific PCR reaction. The primers were examined with DNA isolated from different strains of *Xcc*, different pathovar of *X. campestris* and other strains of *Xanthomonas* isolated from crucifers. Positive results were obtained only with *Xcc* and not with other strains of *X. campestris* or non pathogenic isolates (Fig. 2).

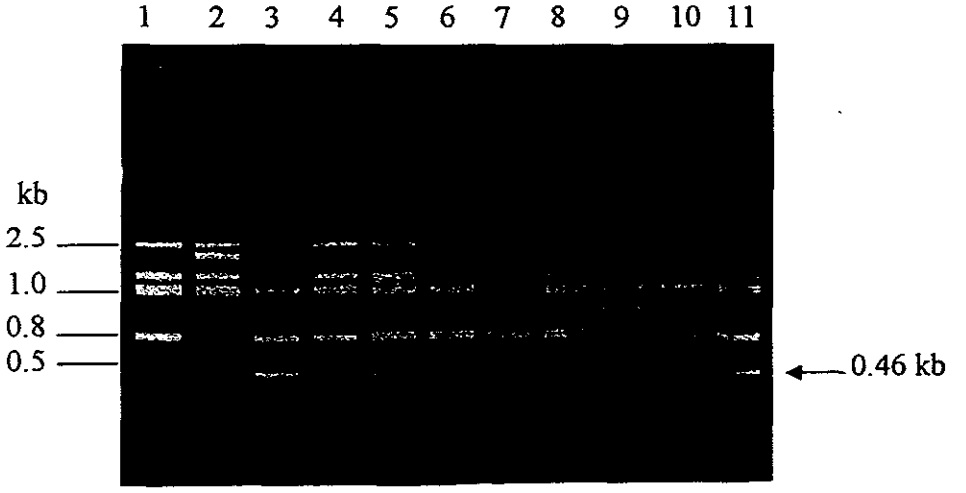

Fig 1: RAPD patterns of different *Xcc* strains generated with primer B13.
#3,4,5,6,10 and 11 represent pathogenic strains whereas the rest are non-pathogenic.

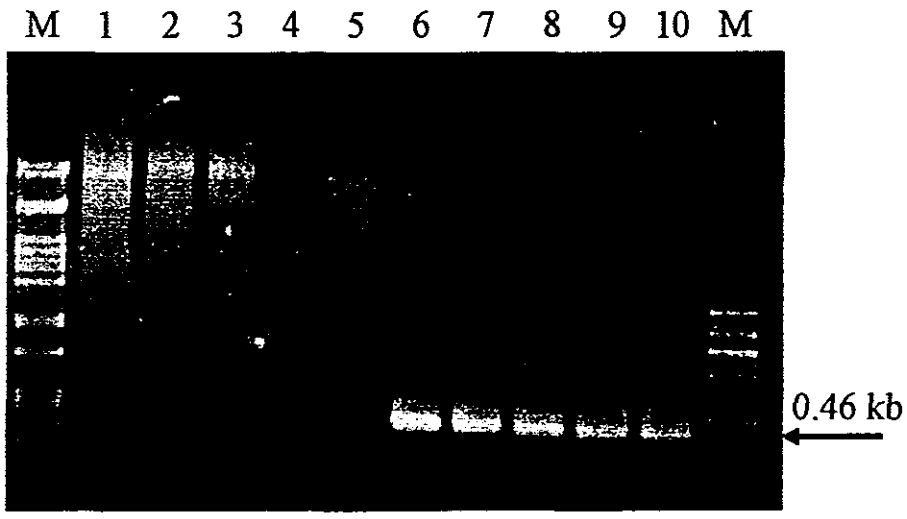

Fig2: PCR reaction carried out with different strains of *Xanthomonas campestris*. M- molecular markers; #1-5 different pathovars of *X. campestris*; 6-*Xcc* isolated in Israel; 7-10 *Xcc* isolated in Kazakhstan.

The threshold of the PCR reaction was determined by adding different concentrations of *Xcc* to extract of cabbage seeds. Non-inoculated seeds (3.7g) were suspended in 15 ml PBS buffer/Tween solution. The suspension was shaken for 15 min and then different concentration of *Xcc* (between 10^8 to 10^2) were added to the extract. One ml of each concentration was centrifuged and the pellet was re-suspended in 500 µl water. Fifty microliters were plated on NSCAA medium for counting the CFU and 10 µl were taken for PCR. Results presented in Fig. 3 demonstrate that about 2 bacterial cells could be detected by this procedure.

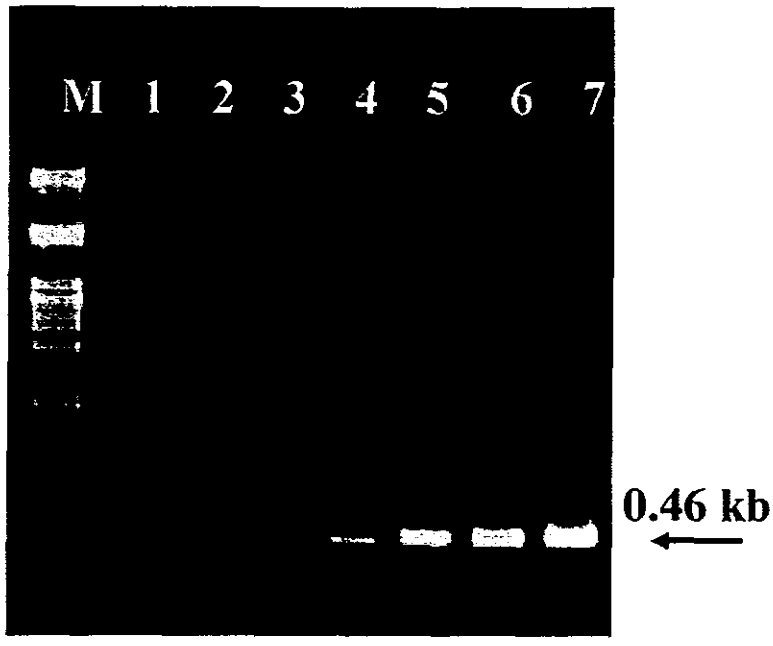

Fig. 3: PCR reaction carried out with different cell concentrations of *Xcc*
M- molecular markers; Lane numbers 1 to 7 represent different cell concentrations in the range of $2x10^2 - 2x10^8$ CFU/ml.

Detection of *Xcc* in cabbage seeds by PCR

During the last year we have examined the use of the PCR procedure for detecting *Xcc* in cabbage seeds. Since naturally infected seeds were not available regularly we carried out the experiments with artificially inoculated cabbage seeds. Cabbage seeds without any chemical treatment were infiltrated with *Xcc* by suspending them in PBS buffer/Tween20 containing different concentrations of *Xcc* under vacuum for 30 min. The infected seeds were allowed to dry in a laminar flow hood for 2 hours and stored until used. For detecting *Xcc*, 1000 seeds (about 3.7g) were suspended in PBS buffer/Tween20 in a ratio of 1:4. The suspension was shaken for 15 min and then 1 ml was removed and centrifuged. The pellet was re-suspended in 100 µl of water. Ten-fold serial dilutions were plated on nutrient agar (NA) plates and NSCAA plates. Ten µl were taken for direct PCR. For Bio-PCR, total colonies grown on NA or NSCAA plates were suspended in 5 ml of water and 10 µl were used per reaction.

The *Xcc* level in the inoculated cabbage seeds was determined immediately after drying the seeds and then after 1, 2 and 7 days. Results presented in Table 2 showed that a slightly higher CFU were obtained on NA plates as compared to NSCAA plates. It was possible to observed bacterial growth after a week with low inoculation level of 10^5

CFU/ml but not with 10^4 CFU/ml. Positive results were obtained by direct PCR with all the other samples. Bio-PCR was less sensitive than direct PCR (Table 1). The latter result can be explained by the difference between the two methods. While Bio-PCR detects only viable cells, direct PCR detects also the dead bacteria.

Table 2: Detection of *Xcc* in artificially inoculated cabbage seeds by PCR procedure

Xcc concentration	Time after inoculation* (days)			
(CFU/ml)	**0**	**1**	**2**	**7**
NA plates				
10^9	$1.4x10^{10}$**	$1.9x10^7$	$7.2x10^6$	$4.8x10^5$
10^7	$8x10^6$	$6x10^5$	$2.4x10^5$	$2.4x10^4$
10^5	$4x10^4$	$1.2x10^4$	$1x10^4$	$6x10^2$
10^4	$1.2x10^3$	10	-	-
NSCAA plates				
10^9	$9.4x10^9$	$1.4x10^7$	$3.1x10^6$	$1.5x10^5$
10^7	$2.2 x10^5$	$4x10^4$	$1x10^4$	$1.6x10^3$
10^5	$8x10^3$	$7.2x10^3$	$2x10^3$	$2.4x10^2$
10^4	$8x10^2$	-	-	-
Direct PCR				
10^9	+	+	+	+
10^7	+	+	+	+
10^5	+	+	+	+
10^4	+	+	-	-
Bio-PCR				
10^9	+	+	+	+
10^7	+	+	+	+
10^5	+	+	+	-
10^4	+	-	-	-

*Seeds were infiltrated with various concentrations of *Xcc* cells.

**Number of bacterial cells per g of seeds

To determine the minimal threshold of infested seeds that can be detected by PCR seeds were infiltrated with 10^7 or 10^8 cells/ml. After 48 hours 1 or 10 inoculated seeds were mixed with 1000 clean seeds and examined as described above. Results obtained showed that it was possible to detect 6-7 CFU/ml or 90-110 CFU/1000 seeds with bio-PCR and 9-10 CFU/1000 with direct PCR.

The PCR protocol for detection of *Xcc* in crucifers seeds is currently being tested with naturally infested seeds in the Official Seed Testing Laboratory at the Volcani Center and in Kazakhstan.

C. Development of a detection method for *Ecc* in cabbage seeds

Ecc strains in Israel were isolated on crystal violet pectate medium (CVP). The strains were tested for Gram reaction, oxidase, nitrat reduction and anaerobic growth. These strains were than subjected to PCR analysis using the primers derived from a specific *pel* gene present in *Ecc* (Darrasse et al. 1994). These primers were confirmed by us to be specific for *Ecc*. Results in Table 3 indicate high correlation between typical growth on CVP and positive reaction with PCR. Thus only 2 isolates out of 38, which were positive on CVP, gave negative result with PCR. Since CVP test is based on pectate degradation and cannot distinguish between *Ecc* and other pectolytic erwinias, it can be concluded that the isolated bacterial population in Israel was mainly composed of *Ecc*. In contrast to Israel, isolation of *Erwinia* in Kazakhstan was carried out on PDA followed by a maceration test with potato cubes. Results shown in Table 3 indicate that more than 50% of the isolates were CVP and PCR negative and therefore could not be identified as *Ecc*. It can be concluded that the CVP is a preferred medium for initial isolation of *Ecc*. This medium has now been replaced the PDA medium used in Kazakhstan.

Table 3: Strains of *Erwinia carotovora* sub. *carotovora* used in this study

Strains	Location	CVP[a]	PCR[b]
Eccp2, Ec322, EcT1, R1, R2, R3, R4, R6, R7, R10, R11, R13, R14, R15, R16, R18, R24, R26, R29, R32, R34, R36, R38, R39, R40, 1639-1/LE, 1537-1/LE, R9, R33, R35, R37, R53, R54, R55, 7/8, 405, 415	Israel	+	+
4K, 5K, 6K, 7K, 8K, 9K, 10K, 11K, 12K, 4bK, 9bK, 25K, 28K, 93K, 103K, 114K	Kazakhstan	+	+
1843/LE, R17, R30, R101	Israel	+	-
95/1, 90, 44, 62, 22-1, 43, 6a, 68, 46, 331, 66, 64, 1/1, 16/1, 186, 93, 416, 16, 98a, 40, 116, 28, 80, 12	Kazakhstan	-	-

[a]Isolates were tested on crystal violet pectate medium (CVP). Positive reaction (+) indicated halo surrounding the colony due to pectate degradation.

[b]PCR was carried out with primers derived from the pectate lyase gene

Detection of *Ecc* in cabbage seeds by PCR

Results presented indicate that the PCR procedure based on *pel* primers can identify strains of *Ecc* isolated from Israel and Kazakhstan. We therefore, examined the use of the PCR procedure for detecting *Ecc* in cabbage seeds.

Cabbage seeds without chemical treatment were inoculated with *Ecc* by suspending them in PBS buffer + Tween 20 (0.05 ml per 100 ml) containing different concentration of *Ecc*, under vacuum for 30 min. The infected seeds were allowed to dry in a laminar flow hood for 2 hours and stored until used. For detecting *Ecc*, 1000 seeds (about 2.1g) were suspended in PBS buffer+Tween in a ratio of 1:4. The suspension was shaken for 15 min and then 1 ml was centrifuged and the pellet resuspended in 100 µl of water. Ten-fold serial dilutions were plated on nutrient agar (NA) plates and crystal violet pectate (CVP) plates. Ten µl were taken for direct PCR. For Bio-PCR total colonies grown on NA or CVP plates were suspended in water and 10 µl were used per reaction. The detection level of the bacteria was determined immediately after drying the seeds and then after 1, 2 and 7 days. Results presented in table 1 showed that on plates no significant differences were observed between NA and CVP media. It was possible to observed growth of bacteria after a week even with low level of inoculation. Positive

results were obtained by PCR with all the samples. Bio-PCR was less sensitive than direct PCR (Table 4). In order to determine the minimal threshold of infested seeds that can be detected by PCR, experiments with dilution of infested seeds were carried out. Results presented in table 5 demonstrate that by direct PCR it was possible to detect 1 infested seed in a mixture with 1000 pathogen-free seeds as compared with Bio-PCR in which the threshold was 10 infected seeds.

Table 4: Detection of *Ecc* in artificially inoculated cabbage seeds by PCR procedure

Ecc **concentration**	**Time after inoculation* (days)**			
(CFU/ml)	**0**	**1**	**2**	**7**
NA plates				
10^8	$3.4x10^{6**}$	$5.2x10^5$	$2.0x10^5$	$3.4x10^5$
10^7	$1.2x10^5$	$8.8x10^4$	$2.4x10^4$	$4x10^5$
10^6	$4x10^4$	$1.2x10^4$	$1x10^4$	$6x10^2$
10^5	$4x10^3$	$1x10^3$	$5x10^2$	$4x10^1$
CVP plates				
10^8	$2.4x10^6$	$4.8x10^5$	$1.7x10^5$	$1.8x10^4$
10^7	$1 x10^5$	$3.8x10^4$	$2.4x10^4$	$4x10^5$
10^6	$4x10^4$	$1.2x10^4$	$1x10^4$	$6x10^2$
10^5	$2.3x10^3$	$1x10^3$	$5x10^2$	$4x10^1$
Direct PCR				
10^8	+	+	+	+
10^7	+	+	+	+
10^6	+	+	+	+
10^5	+	+	+	+
Bio-PCR				
10^8	+	+	+	+
10^7	+	+	+	+
10^6	+	+	+	-
10^5	+	+	-	-

*Seeds were infiltrated with various concentration of *Ecc* cells.

**Number of bacterial cells per g of seeds

Table 5: The minimal threshold of infested seeds detected by PCR procedure

Infested seeds*	NA plates		CVP plates		Direct PCR		Bio-PCR	
	0	7 days	0	7 days	0	7 days	0	7 days
100	$4.5x10^5$	$8.5x10^3$	$7.3x10^5$	$4.5x10^3$	+	+	+	+
50	$2.6x10^5$	$8.9x10^3$	$2.3x10^5$	$6.5x10^2$	+	+	+	+
10	$4.5x10^4$	$6.9x10^3$	$4.1x10^4$	$4.0x10^2$	+	+	+	+
1	$2.2x10^4$	$2.8x10^3$	$5.7x10^3$	$1.2x10^3$	+	+	-	-

*Seeds were infiltrated with 10^9 cells /ml. Infested seeds were diluted with 1000 pathogen-free seeds.

Impact Relevance and Technology Transfer

The ultimate goal of this project was to introduce modern diagnostic procedures for phytobacterial pathogens to Kazakhstan. Prior to this project identification of bacterial pathogens in this country relied only on characteristic symptoms in the filed, isolation of bacterial colonies on simple media and pathogenicity tests. Needless to add that these procedures are time consuming and are not specific and sensitive enough for accurate identification. The introduction of PCR-based procedures for detection of microorganisms revolutionized the diagnostic approaches and is now well established in advanced laboratories. Therefore emphasis was given to bring up to date the diagnostic methods of bacteria used in Kazakhstan. Training the Kazakh scientists on one hand and renovating the laboratories with new equipment and necessary materials achieved this purpose.

Xcc and *Ecc* have been selected for practicing the new methods because of their economic importance in Kazakhstan, Israel and worldwide. During the period of this project a collection of *Xcc* and *Ecc* strains was made by the Kazak scientist. This necessitated traveling to different regions of Kazakhstan, which allowed the Kazakh researches to evaluate the extent of the damage caused by *Xcc* and *Ecc* as well as by other plant pathogens. The training included two laboratories in Kazakhstan from different institutes (i.e. National Academy of Sciences - Institute of Microbiology and Virology and Kazakh Agricultural Institute in Almaty). Since these bacterial pathogens are also prevalent and important in Israel it facilitated the development of the PCR

procedures in our laboratory at the Volcani Institute which also served as the training place for the Kazakh scientists.

As a result of the project the laboratory in the Institute of Microbiology and Virology in Kazakhstan is well equipped and capable of performing the above mentioned the PCR diagnostic procedures. Obviously the impact of these achievements in the future will depend on adequate organization by the government or private sectors which will use the obtained knowledge for establishing a routine clinical laboratory.

Project Activities

During the course of this project a constant communication was maintained by frequent e-mails and telephone calls. During 1998 Dr. Nazira Ajtkhozhina of the National Academy of Sciences visited Israel and was first exposed to PCR and other procedures necessary for achieving the project goals. On June 1998 Drs. Shulamit Manulis and Isaac Barash visited Kazakhstan in order to help organizing the work in both institutes and reviewed the various procedures involved in isolation and characterization of the pathogens. Dr. Aliya Dzaimurzina visited Israel at the end of the same year. She was exposed to improved semi-selective media and biochemical methods for identification of the pathogens as well as PCR method. In 2000 Dr. Nazira Ajtkhozhina visited Israel again and participated in the strategy and work conducted in Volcani Center for developing the specific PCR procedure for *Xcc*. In addition, she transferred chemicals and small equipments purchased in Israel to Kazakhstan.

A common scientific report will be presented at the International congress of Plant Pathology that will be held in 2003 in New Zealand. The results are also being summarized in a common manuscript that will be submitted during 2003.

Project Productivity

The project accomplished the first proposed stage namely introduction of modern diagnostic procedures of phytopathogenic bacteria in Kazakhstan. Achievement of the next stage, namely the utilization of the developed protocols for routine work will depend on the Kazakh scientists as mentioned above.

Future Work

The work on diagnosis has achieved its goals and it can only be followed by advising the Kazakh scientists how to extend it further to other plant pathogens. Future work can

be aimed at studying mode of pathogenicity by these or other pathogens in order to elevate the level of research in plant pathology in Kazakhstan.

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